

***Tulostoma rufescens* sp. nov. from Sonora, Mexico**

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ABSTRACT—A new species of stalked puffball, *Tulostoma rufescens*, was observed and collected from subtropical scrub vegetation within the Sonoran Sky Islands, Mexico, and was characterized morphologically and molecularly. The new fungus is characterized by small to medium sized spore-sacs, a thinly membranous exoperidium persisting in patches in the pinkish endoperidium, a tortuous stem with a basal bulb strongly intermixed with sand and debris, subhyaline capillitia with swollen and pigmented septa, and strongly echinulate basidiospores with spines that occasionally coalesce to form a subreticulum. Maximum likelihood and neighbor-joining phylogenetic analyses of full ITS1-5.8S-ITS2 and D1-D2 LSU DNA regions placed our collection within the monophyletic genus *Tulostoma* but separate from all of the available sequenced species.

KEY WORDS—*Agaricaceae*, *Agaricales*, chorology, gasteroid fungi, molecular systematics

Introduction

The Madrean Sky Islands (or Madrean Archipelago) are a set of approximately 40 mountains in southern and southeastern Arizona, southwestern New Mexico, and northwestern Mexico. They combine temperate pine-oak forests at the highest elevations with arid to semiarid vegetation in the lowlands, the latter forming part of the Sonoran and Chihuahuan deserts. These environments preserve a remarkable richness, complexity, unusual neo- and archaeo-endemics, and an exceptional mixture of Nearctic and Neotropical

species (Warshall 1995). The biodiversity of the Madrean Sky Islands has been studied extensively, totaling close to two thousand species including 1380 plants, 358 birds, 104 reptiles and amphibians, 76 mammals, and 39 fish species (Van Devender & al. 2013), but information on fungal diversity is scant.

Sonora is the second largest Mexican state (179,355 km²) and ranks fifth of the 32 states of Mexico in fungal diversity with more than 618 morphospecies (Aguirre-Acosta & al. 2014), of which around 210 represent gasteroid and sequestrate *Agaricomycetes* (GSA), with *Tulostoma* Pers. (with 30 spp.) being the most diverse and representative genus (Hernández-Navarro & al. 2017). Some Sonoran GSA species, including the stalked puffballs, are rare worldwide; e.g., *T. portoricense* is known only from its type locality (Puerto Rico) and Sonora (Esqueda-Valle & al. 1998), and *T. gracilipes* only from its type locality (South Africa) and Sonora (Piña & al. 2010).

Tulostoma was proposed and sanctioned by Persoon (1794, 1801) and is characterized by gastrocarps composed of a hollow stalk inserted in a socket of a spore-sac with an apical ostiole. The genus has a cosmopolitan distribution but is especially richly diverse and abundant in arid and semiarid areas. According to Wright (1987), most species exhibit a terrestrial habit, with the remarkable exception of *T. exasperatum*, which grows in decaying wood. *Tulostoma* species can be classified based on their habitat as psammophilous (sandy soils in arid regions), terricolous (clay-loving species, in pastures, roadsides), and “forest soil-loving species” (in tropical or temperate zones with a high content of organic matter). In his *Tulostoma* world monograph, Wright (1987) included 139 species; at the present time, Species Fungorum accepts c 155 taxa (www.speciesfungorum.org).

There is scant molecular information on *Tulostoma* species. From the Americas, only *T. domingueziae* from Argentina has been described based on molecular data (Hernández Caffot & al. 2011); and from Asia only *T. ahmadii* from Pakistan (Hussain & al. 2016). Jeppson & al. (2017) made a major contribution, sequencing European species of *Tulostoma*, including 34 holotypes and some specimens from other continents. As a result, they characterized 30 known species, proposed five new species, and identified at least 27 new undescribed species; they also confirmed *Tulostoma* as monophyletic, found an unexpectedly large cryptic diversity, and cited areas of steppe vegetation in Hungary and Spain as hot spots for *Tulostoma* species diversity. The complete ITS1-5.8S-ITS2 nrDNA region is considered as the barcode for fungal species recognition. To reconstruct a better phylogeny, it has been suggested to complement this information with other ribosomal regions

such as D1-D2 LSU, and/or protein coding genes such as *atp6*, *EF1- α* , or *RPB1*, among others (Stielow & al. 2015).

As part of a major research, we studied some unidentified *Tulostoma* collections from the Sonoran Sky Islands whose morphological characterization did not match with any of the currently known morphospecies; their molecular characterization led us to propose *T. rufescens* as a new species.

Materials & methods

The studied material is preserved in the Collection of Macromycetes of Sonoran State University, Hermosillo, Mexico (UES). It was collected in sandy soil, in a subtropical scrub vegetation, located at 29°53'44" N 109°27'21" W at 1214 m asl. Basidiomes were characterized and conserved following conventional mycological techniques. Codes in parentheses after colors in basidiomata descriptions follow Kornerup & Wanscher (1978). Microscopic features were measured by examining gleba sections mounted in 10% KOH preparations using an Olympus BX-51 light microscope (LM). Fifty spores from each basidiome (including ornamentation, capillitia, and septa) were randomly measured and the mean and standard deviation were calculated. All measurements were made using the Infinity analyze Software v. 6.5.4 (Lumenera Corp.). Scanning electron microscope (SEM) micrographs were produced with a JEOL-JSM 600 LB microscope using critical point drying and sputtered with gold-palladium according to Moreno & al. (1995).

Genomic DNA was extracted following a standard CTAB 2% protocol (Cubero & al. 1999) with some modifications. After first grinding stipe tissue in liquid nitrogen and placing ~100 mg of dusted tissue into a 2 mL tube, 1 mL CTAB 2% + 100 μ L of β -mercaptoethanol were added, and the tubes were incubated at 55°C for 30 min, mixing every 10 min. Then, 600 μ L of chloroform : isoamyl alcohol (24:1) was added and mixed by inversion for 10 min. The mix was then centrifuged for 15 min at 12,000 $\times$ g; the supernatant was transferred to a 1.5 mL tube and 500 μ L of isopropanol + 50 μ L of 3M sodium acetate was added, mixed by inversion, and stored at -20°C for 1 h. The mix was centrifuged 10 min at 12,000 $\times$ g and the supernatant discarded. The remaining pellet was washed twice with EtOH 70%, dried at room temperature, and resuspended in 50 μ L of ultrapure water. The gDNA was then treated with 1 μ L of 10 mg/mL of RNaseA and stored for 30 min at 37°C, quantified in a NanoDrop™ 2000, and its integrity verified by visualization on a 1% agarose gel stained with Ethidium Bromide (EtBr).

The gDNA was diluted to 10 ng/ μ L to amplify nuclear ribosomal RNA genes (the full ITS1-5.8S-ITS2 and D1-D2 LSU regions); for this, we used the primer pairs ITS1/ITS4 and LR0R/LR5. PCR reactions were carried out in a volume of 20 μ L, with 20 ng gDNA, using the mix content and thermal cycler conditions described by Schoch & al. (2012). PCR amplicons were then visualized in a 1% agarose gel stained with EtBr. Amplicons were purified from the gel using the kit Wizard® SV Gel and PCR Clean-Up System and cloned using pGEM®-T-Easy following the manufacturer's instructions

using *Escherichia coli* DB10B chemically competent cells. The recombinant clones were grown in LB medium with IPTG (100 mM), ampicillin (50 µg/mL), and X-gal (50 mg/mL) as selection markers. The positive colonies were grown overnight in LB broth with ampicillin (50 µg/mL) and the pDNA was extracted using the alkaline lysis method (Sambrook & al. 1989). The presence of the inserts was verified by visualizing EcoRI enzymatic digestion of 1 µg of the pDNA, and also by PCR using the primers M13F and M13R in 1% agarose gels stained with EtBr. The pDNA was sequenced in triplicate by MacroGen Korea.

The obtained sequences were manually curated by inspecting their chromatograms on the SequencherSoftware™ v. 5.2.3 and the clean assembled sequence was used for BLASTN query at NCBI's GenBank. Then, a MegaBlast was performed, and 95 highly similar *Tulostoma* sequences and two outgroup sequences were downloaded from GenBank, aligned using the MUSCLE algorithm with default parameters (Edgar 2004), and manually edited using MEGA 6.0 software suite (Tamura & al. 2013). We performed two different molecular phylogenetic analyses for the 98 complete ITS1-5.8S-ITS2 and D1-D2 LSU rDNA gene sequences. One tree consisted of a Maximum likelihood (ML) phylogenetic analysis, with the GTR+G+I model (Nei & Kumar 2000) with gaps treated as partial deletions with a 95% of coverage, using an NNI heuristic method for topology improvement; the other consisted of a Neighbour-Joining (NJ) distance analysis, with gaps treated as pairwise deletions (Saitou & Nei 1987); both with 1000 bootstrap replicates. The trees were rooted using *Mycenastrum corium* as ingroup for *Agaricaceae* and *Psathyrella secotioides* as outgroup for *Psathyrellaceae* (Matheny & al. 2006, Larsson & Jeppson 2008, Moreno & al. 2015).

Results

Tulostoma rufescens Hern.-Nav. & Esqueda, sp. nov.

FIGS 1–6

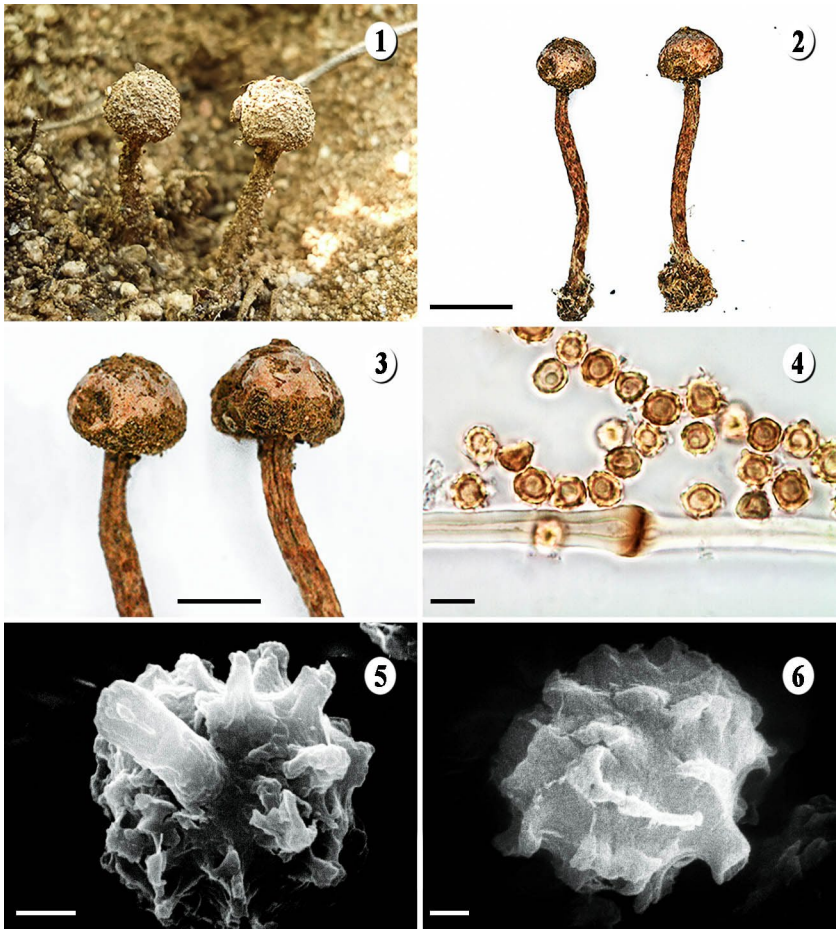
MYCOBANK MB 821805

Differs from *Tulostoma adhaerens* by its small to medium size spore-sacs, thinly membranous exoperidium persisting in patches in the pinkish endoperidium, and strongly echinulate basidiospores with the spines sometimes coalescing to form a subreticulum.

TYPE: Mexico, Sonora, municipality of Moctezuma, “La Madera,” 29°53'44"N 109°27'21"W, alt. 1214 m, sandy soil, in a subtropical scrub vegetation, 2 August 2010, leg. E. Hernández-Navarro, C. Piña, R. Maldonado & A. Gutiérrez (Holotype, UES 10528) (two complete basidiocarps); GenBank MF319226).

ETYMOLOGY: The name refers to the pinkish tone of the endoperidium.

SPORE-SAC 7.5–8.4 mm diam. × 5.7–5.9 mm high. EXOPERIDIUM thinly membranous, easily removable in big scales when dissected, dark because of sand and particles on the outside and whitish in the inside. Persistent in parts of the endoperidium like clay or scales, especially at the basal part of the spore-sac. ENDOPERIDIUM grayish pink (11B3–12B3), mottled with dark spots that



FIGS. 1–6. *Tulostoma rufescens* (holotype, UES 10528): 1–3. Basidiocarps; 4. Capillitium and spores (LM); 5, 6. Spore ornamentation (SEM). Scale bars: 2 = 10 mm; 3 = 5 mm; 4 = 5 μ m; 5, 6 = 1 μ m.

simulate warts due to the effect of the persistent exoperidium. MOUTH round to elliptic, 1 $\times$ 1.2 mm diam. and with a projection up to 0.4 mm in height. SOCKET conspicuous, deep, quite separate from the endoperidium, with a denticulated to lacerated membrane. GLEBA dark ferruginous to brown (8D4–8E8). STIPE partially buried in the sand, woody-fistulose, tortuous, 27–28 $\times$ 1.7–1.8 mm, light brown to reddish brown (6C4–7E8),

surface rugose-scaly, with a conspicuous basal bulb with hyphae strongly mixed with grains of sand and debris. SPORES globose to subglobose, reddish brown, echinulate, some spines slightly curved, some appearing subreticulate, 4.2–6.7 μm including ornamentation [mean = 5.1 μm , $Q_m = 0.99$ $n = 100$]; under SEM the ornamentation is formed by conspicuous conic structures, spines commonly fused in the apex, but some coalescing irregularly to a subreticulate pattern, with a pedicel variable in size. CAPILLITIUM subhyaline to slightly colored, 2–7 μm diam. [mean = 3.9 μm , $n = 100$], septa concolor with the spores, somewhat swollen 3–9.9 μm [mean = 5.5 μm $n = 100$].

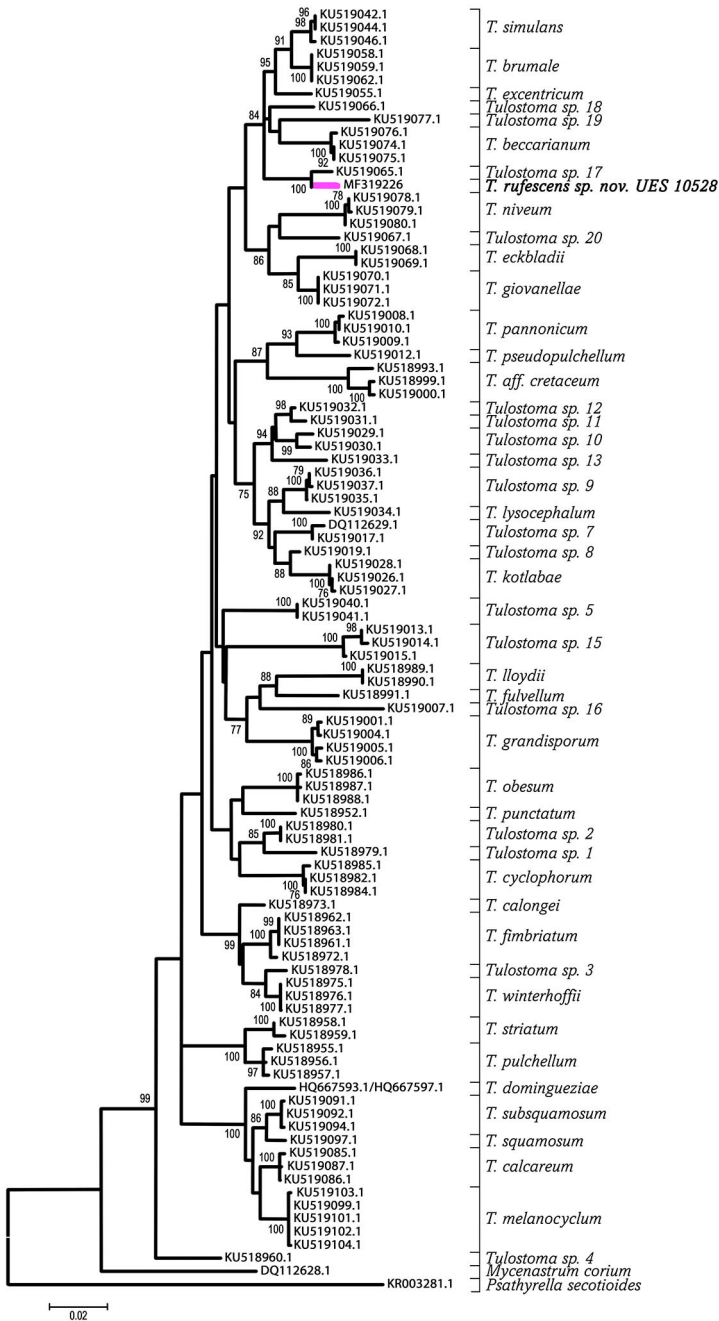
COMMENTS—Initial sequence homology tests by BLASTN of ITS region showed that the closest species is *Tulostoma* sp. 17 (Jeppson & al. 2017), an undescribed species from Hungary and Spain whose ITS barcode differs from our collection by 4% (data not shown). ML analyses of the complete ITS-5.8S-ITS and D1-D2 LSU regions placed our collection within the monophyletic genus *Tulostoma* (FIG. 7) and support its separation from similar morphospecies (e.g., *T. squamosum*).

When using distances in an NJ tree, the results are similar (FIG. 8). Some changes can be observed, especially concerning unnamed taxa and topology of lower branches. *Tulostoma* sp. 17 has not been described and its morphological traits are unknown; nonetheless, molecular analysis suggests that our collection is different from any sequenced *Tulostoma* species to date. It was also remarkable how the treatment of the gaps gave a better topology for NJ analysis than ML. This same result was observed with other algorithms (UPGMA, Minimum Evolution, Maximum Parsimony) when gaps were treated as pairwise deletions (data not shown).

Discussion

Tulostoma rufescens is distinguished by combination of the following characters: small to medium size spore-sac (<9 mm), pinkish endoperidium (a very uncommon color in the genus), tortuous stem with a conspicuous basal bulb, and the strongly echinulate spores that may coalesce to form a subreticulum in some spores.

FIG. 7. Molecular phylogenetic analysis of *Tulostoma* species based on the Maximum Likelihood method using the General Time Reversible model. The tree with the highest log likelihood (–8278.3120) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches, based on 1000 bootstrap replicates. The numbers after “*Tulostoma* sp.” refer to Jeppson & al. (2017). Our new sequence is set in bold.



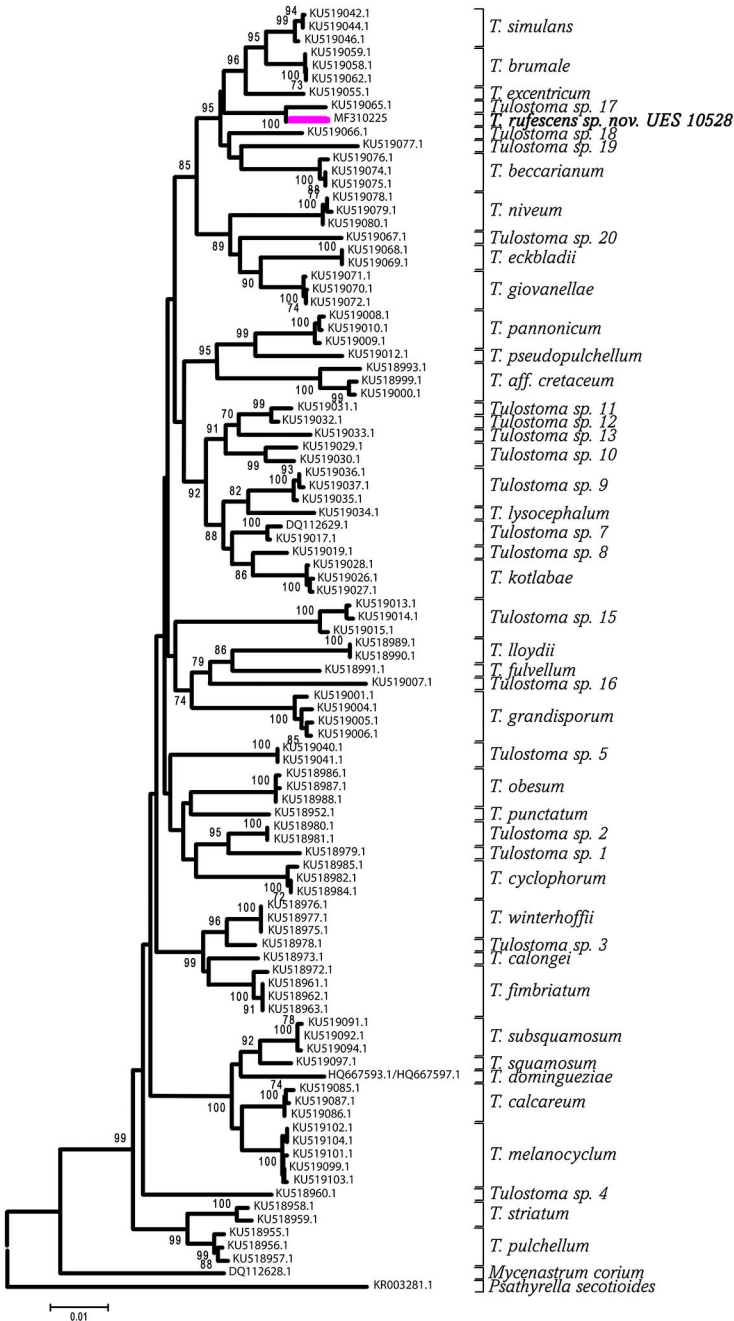
The closest morphospecies is possibly *T. adhaerens* Lloyd recorded from South Africa, Madagascar, Australia, Malaysia, and Japan, which differs by its indistinct exoperidium, more robust sporocarp, bigger spores (5.0–7.5 µm diam.), and episore comprising independent spines, some of which are at the apex. Wright (1987) described the Australian holotype and isotype of *T. adhaerens* as medium sized (10–20 mm) with an indistinct exoperidium and subreticulate spores under LM but echinulate with independent spines in SEM. The Japanese material is close to 15 mm diam., with a spore ornamentation comprising several spines in coherent fascicles and each spine isolated at the base (Asai & Asai 2008). Wright (1987) designated *T. adhaerens* as the type of *Tulostoma* sect. *Hyphales* J.E. Wright based on its exoperidium with a thick layer of hyphae and a tubular or compressed cylindrical ostiole. Of the 37 species included in this section, 35 are accepted in Species Fungorum.

Tulostoma rufescens can also be confused with *T. squamosum* (J.F. Gmel.) Pers., which differs by its true verrucose exoperidium formed by irregular dark cells with a thick wall (sphaerocysts) and a squamous reddish brown stipe. A similar case would be *T. subsquamosum* Long & S. Ahmad, which also has spines but truly subreticulate spores in both LM and SEM, and an uncolored to ochraceous endoperidium. *Tulostoma rufescens* could also be confused with *T. beccarianum* Bres. and allied species such as *T. simulans* Lloyd, two taxa once synonymized based on morphology, but recently supported as independent by molecular data. The two species are separated morphologically from *T. rufescens* by their uncolored endoperidium and smaller spores with verrucae, not spines (Altés & Moreno 1993, Altés & al. 1996, Jeppson & al. 2017).

Tulostoma rufescens might be confused with *T. calcareum* Jeppson & al., which differs by its hyphal-verrucose exoperidium that is deciduous (but sometimes persisting as whitish scattered verrucae); its brownish-ochraceous endoperidium, initially rather dark colored but with age fading to greyish white; its greyish to brownish peristome; and its slightly smaller verrucose-echinate spores (4–6 µm, mean = 4.7–5.0 µm; Jeppson & al. 2017).

There is a debate about gap management in phylogenetic analyses. It has been reported that treating indels as missing data in both Bayesian

FIG. 8. Molecular phylogenetic analysis of *Tulostoma* species based on the Neighbor-Joining method. The optimal tree is shown (with the sum of branch length = 0.89290951). The percentage of trees in which the associated taxa clustered together is shown next to the branches, based on 1000 bootstrap replicates. The numbers after "*Tulostoma* sp." refer to Jeppson & al. (2017). Our new sequence is set in bold.



and maximum likelihood phylogenetic estimations can be statistically inconsistent for determining a general and rather simple model of sequence evolution. Resulting priors on branch lengths and rate heterogeneity parameters may exacerbate the effects of ambiguous data, producing strongly misleading bipartition posterior probabilities, even while showing the true alignment (Nagy & al. 2012).

Some authors use a 3% threshold of sequence identity to determine conspecificity (Begerow & al. 2010); nevertheless, this value is not generally accepted for all fungi, since it has been shown to vary between groups, being too high for some taxa and too low for others (Nilsson & al. 2008). In both our analyses, bootstrap values were strong ($\geq 70\%$) in the higher branches, while some lower branches were not well supported ($\leq 70\%$). Low bootstrap values are related to evolutionary processes like incomplete lineage sorting and introgression of alleles across species boundaries (e.g. incomplete reproductive isolation) due to different selective processes (Morando & al. 2004).

Balasundaram & al. (2015) have suggested that the right DNA marker (or a particular combination of markers) and its intraspecific distances must be evaluated in order to reconstruct the accurate phylogeny of each group. The interspecific genetic distances must also be evaluated, as such distances are higher in geographically widely distributed species, stressing the importance of sampling more specimens from wider geographical ranges to determine intraspecific variation. In the absence of this information, the proposal of new names might not reflect cryptic diversification but rather taxonomic inflation, leading to a changed species concept rather than to new discoveries. This directly influences macroecology and conservation analyses, most of which are based on species lists (Isaac & al. 2004).

In addition, many researchers admit that the ITS region does not provide a precise species recognition for some fungal groups, such as for yeast, arbuscular mycorrhizas, and lichens. The two spacer regions (ITS1, ITS2) do not evolve independently because the variation of both regions is highly correlated, and 5.8S rRNA region is well conserved within all fungal species. However, the complete ITS meets the criterion for a good barcode marker: it is short (< 1000 bp), and there are several copies in the genome, making it easily amplified, even from degraded, environmental or old herbarium samples (Schoch & al. 2012). Other protein-coding genes might give a better species resolution but lack the many practical applications of ITS (Köljalg & al. 2013). The ITS region is repeatedly criticized for indel-induced alignment

problems and the lack of phylogenetic resolution above the species level, one reason why some authors chose to delete ambiguous characters from ITS and thereby losing potentially valuable information. However, it has been argued that ITS indels are slightly more conserved than nucleotide substitutions and when included in phylogenetic analyses improved the resolution and branch support, thus extending the resolving power of ITS (Nagy & al. 2012).

From a barcoding perspective and considering only the complete ITS region, currently fewer than 40 *Tulostoma* species have been properly sequenced, representing approximately 25% of known morphospecies. Despite the unusual characteristics of our collection, there are several examples of convergent evolution in fungi, where the molecular characterization of specimens has revealed cryptic species with shared morphological traits (Nguyen & al. 2013, 2016). *Tulostoma* is no exception, since common species such as *T. fimbriatum* are polyphyletic, supporting the idea that many of the morphological characters used for segregation of taxa are plesiomorphic or homoplastic due to convergent evolution or parallelism (Jeppson & al. 2017).

Nevertheless, further molecular analysis of the holotype of *T. adhaerens*, other *Tulostoma* holotypes, and *Tulostoma* specimens from Sonora and elsewhere are needed so as to verify the authenticity of names based on morphological species concepts, estimate the actual number of species, determine fully which morphological traits are taxonomically and phylogenetically informative, and understand the boundaries between closely-related species and genera.

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